IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Ronald B. Gartenhaus Examiner: Hong Sang

Scrial No.: 09/709,131 Art Unit: 1643

Filed: November 10, 2000

For: MCT-1, A Human Oncogene

37 C.F.R. 1.132 Declaration

Commissioncr for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

The undersigned, Ronald B. Gartenhaus, declares that:

1. I am the inventor on the above-referencd patent applieation.

2. I am an author on the referenee of Dierov et al., *Increased G1 Cyclin/cdk Activity in Cells Overexpressing the Candidate Oncogene, MCT-1*, Journal of Cellular Bioehemistry 74:544–550 (1999).

3. All of the authors other than me listed on the Dierov et al. referenee eited above were working under my supervision and did not make an inventive contribution to the invention.

4. All statements madc herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 101 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued therefrom.

Respectfully submitted,

4/8/10

Date

Dr. Ronald B. Gartenhaus

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2. I am an author on the reference of Dierov et al., *Increased G1 Cyclin/cdk Activity in Cells Overexpressing the Candidate Oncogene, MCT-1* (Journal of Cellular Biochemistry 74:544–550 (1999)), ("Dierov" hereafter), a copy of which is appended hereto as Exhibit A.

3. I invented antibodies that bind with specificity to MCT-1 protein at least as early as January 26, 1999. This is evident from the description of the development of antibodies to MCT-1 (see page 546 under "Antibodies"), and from Fig. 1B in Dierov. The description of antibodies specific for MCT-1 in Dierov refers to antibodies that specifically recognize MCT-1 protein that contained the same amino acid sequence as SEQ ID NO:8 in the above-refereneed patent application. The immune sera provided by Research Genetics (described on page 546 under "Antibodies" of Dierov) was made at my direction and no one at Research Genetics made any inventive contribution to production of the sera. Dierov was accepted for publication by the *Journal of Cellular*

Biochemistry on January 26, 1999 (see bottom left of p545). The same description of the development of antibodies to MCT-1 protein and Fig. 1B that appears in Dierov was in the manuscript accepted for publication on March 4, 1999 (see bottom left of p545). Therefore, Dierov is evidence that I invented antibodies that bind with specificity to MCT-1 protein at least as early as January 26, 1999.

4. All statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 101 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued therefrom.

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Date

Dr. Ronald B. Gartenhaus

EXHIBIT A

Journal of Cellular Biochemistry 74:544–550 (1999)

Increased G1 Cyclin/cdk Activity in Cells Overexpressing the Candidate Oncogene, *MCT-1*

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Abstract We have recently identified a novel candidate oncogene, *MCT-1*, in the HUT 78 T-cell line. When overexpressed in NIH3T3 fibroblasts, the *MCT-1* gene shortens the G1 phase of the cell cycle and promotes anchorage-independent growth. Progression of cells through a late G1 phase restriction point is regulated by G1 cyclins whose phosphorylation of the retinoblastoma gene product facilitates entry into S phase. Deregulated expression of G1 cyclins and their cognate cdk partners is often found in human tumor cells. In order to address the potential relationship of *MCT-1* to cell cycle regulatory molecules, we analyzed the ability of *MCT-1* overexpression to modulate cdk4 and cdk6 kinase activity in NIH3T3 fibroblasts constitutively overexpressing *MCT-1*. We observed an increase in the kinase activity of both cdk4 and cdk6 in asynchronously growing transformed cells compared with the parent cells. This increased kinase activity was accompanied by an elevated level of cyclin D1 protein and increased G1 cyclin/cdk complex formation. We also observed a correlation between increased protein levels of MCT-1 with cyclin D1 expression in a panel of lymphoid cell lines derived from T-cell malignancies. These results demonstrate that constitutive expression of MCT-1 is associated with deregulation of protein kinase-mediated G1 phase checkpoints. J. Cell. Biochem. 74:544–550, 1999.

Key words: *MCT-1* gene; G1 cyclins; kinase activity

Tumorgenesis is a multistep process with a causal relationship between the accumulation of genetic abnormalities and more aggressive clinical behavior [Fearon et al., 1990; Califano et al., 1996]. In many tumors, amplification of critical growth-inducing genes is frequently observed with the abrogation of G1 phase checkpoints [Lammi et al., 1991; Motokura et al., 1991]. We have recently isolated *MCT-1*, a novel candidate oncogene using the arbitrarily primed-polymerase chain reaction method (AP-PCR) [Prosniak et al., 1998]. There was a region of sequence homology between the N-terminus of the *MCT-1* predicted polypeptide and the C-terminal region of cyclin H [Prosniak et al., 1998], a region important for protein–protein interactions [Andersen et al., 1997]. Overexpression of the *MCT-1* gene significantly shortened cell doubling time and transformed NIH3T3 fibroblasts in vitro. This was accompanied by a significant reduction in the G1 phase of the cell cycle, consistent with the involvement of *MCT-1* in cell cycle progression [Prosniak et al., 1998]. Distinct cyclin/cdk complexes are formed at different phases of the cell cycle with activation of their cognate kinase activities. Progression of cells through a late G1 phase restriction point is regulated by G1 cyclins whose phosphorylation of the retinoblastoma and related gene products facilitates entry into S phase [Sherr, 1994]. Deregulated expression of G1 cyclins and their cognate cdk partners is often found in human tumor cells [Lammie et al., 1991; Motokura et al., 1991]. We speculated that MCT-1 might have an effect on protein kinase-mediated G1 phase checkpoints. A common perturbation in G1-associated cyclins is overexpression of the cyclin D1 molecule, which has been shown to contribute to the oncogenic transformation of cells both in vivo and in vitro [Jiang et al., 1993; Lovec et al., 1993]. In order to address the potential relationship that *MCT-1* might have with cell cycle regulatory molecules, we analyzed the ability of *MCT-1* overexpression to modulate cdk4 and cdk6 kinase activity in NIH3T3 fibroblasts con-

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stitutively overexpressing *MCT-1*. We also examined the levels and composition of the G1-S phase cyclin/cdk complexes in transformed fibroblasts.

MATERIALS AND METHODS

NIH 3T3 Cell Culture and DNA Transfection

Stably transfected NIH3T3 cell lines constitutively expressing *MCT-1* (pCMV-*MCT-1*) or vector control (pCMV) transfected NIH3T3 cell lines were selected after growing in DMEM supplemented with 10% heat-inactivated calf serum, penicillin, streptomycin and 1,000 μg/ml geneticin (G418) (Gibco, Grand Island, NY) for 2 weeks, as previously described [Prosniak et al., 1998]. Individual clones of transfected cells were obtained by limiting dilution. Transient assays were performed using the Lipofectamine method according to the supplier's instructions (GIBCO). Briefly, an expression vector with an HA tag at the N-terminus of *MCT-1* (pcDNA-HA-*MCT-1*) was constructed by cloning into pcDNA3-HA the coding cDNA for *MCT-1* into the *Bam*H1 and *Eco*R1 sites, creating a fusion protein HA-*MCT-1*. Transfected cells were analyzed 48 h later for the fusion protein HA-*MCT-1*.

Lymphocyte Cell Lines

PBL (peripheral blood lymphocytes) were prepared from whole fresh blood of healthy donors. Mononuclear cells were isolated by centrifugation over Ficoll (Organon Teknika, Durham, NC) cultured for 48 h in RPMI 1640 medium containing 20% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 1.0% phytohemagglutinin (PHA) (Gibco; Grand Island, NY). The nonadherent cells, PBL were viably frozen for further analysis.

IL-2-independent cell lines included C10MJ, MT-2, Hut 78, H-9, HUT 102, DA 202, and C91PL (Advanced Biotechnologies, Columbia, MD). They were all grown in RPMI 1640 medium containing 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mmol/L glutamine (Gibco). IL-2-dependent cell lines N1185 and N1186 [Berneman et al., 1992] were grown in the same culture conditions as above with the addition of recombinant interleukin-2 (IL-2) (40 U/ml) (Gibco).

Immunoprecipitation and Immunoblotting

Cell pellets were lysed with lysate buffer; 10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, and 150 mM PMSF. Total protein concentration in each sample was determined using a micro BCA method (Pierce, Rockford, IL) according to the manufacturer's instructions. Equal amounts of whole cell lysate (50–100 μg) was resuspended in 5 ml TBS containing 1 μg^{-ml} leupeptin, 1 μg/ml aprotinin, 0.01% PMSF, 0.01% TPCK, 0.01% TLCK, 0.1% Na Azide and 1% NP-40 (Sigma, St. Louis, MO). Samples were precleared with Protein-G beads (Gibco) and either normal rabbit or mouse serum (1:1,000 dilution). Immunoprecipitation with cyclin D1 was carried out for 12 h at 4°C. Immune complexes were precipitated with 1–5 μg antibody and Protein-G agarose then heated at 95°C for 5 min in sample buffer containing; 93 mM Tris pH 6.8, 3% SDS, 1.1 mM B-mercaptoethanol 0.03% BPB, and 15% Glycerol (Sigma). Eluant was analyzed on a denaturing, reducing SDS-PAGE and transferred to supported nitrocellulose paper by electroblotting. Filters were incubated with (1–5 μg) one of the following antibodies; cyclin D1, cdk4, cdk6, PCNA, and p21. Secondary antibody used was either an anti-mouse or anti-rabbit horseradish peroxidase (HRP)-linked whole antibody. Chemiluminescence was then performed with ECL (Amersham Life Science, Arlington Heights, IL) according to the manufacturer's instruction.

Immune Complex Protein Kinase (CDK4 and CDK6) Assay

Pellets from NIH3T3 cells were lysed with lysate buffer; 10 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, and 150 mM PMSF. Total protein concentration in each sample was determined using a micro BCA method (Pierce, Rockford, IL) according to the manufacturer's instructions. We then transferred a 50-μg cell extract to microfuge tube (total volume in 500 μl IP buffer). Immunoprecipitations were carried out by incubation overnight at 4°C with 2.5 μg of mouse monoclonal cdk4 antibody or cdk6 antibody, followed by incubation for 4 h with 25 ml of protein G-agarose beads. Precipitated protein pellets were washed 3 times with ice-cold kinase lysis buffer and then resuspended in 20 ml of ice-cold kinase buffer [50 mM HEPES (pH 7.5), 80 mM β-glycerophosphate, 2.5 mM ethyleneglycol bis (b-aminoethyl ether)-N,N,N ,N -tetraacetic acid (EGTA), 10 mM $MgCl_2$, 1 mM DTT, 2.5 mM phenylmethylsulfonyl fluoride, 60 KIU/ml aprotinin, 10 μg/ml leupeptin, 10 mm cyclin AMP-dependent protein kinase-inhibi-

tory peptide] (Sigma). A 12-ml of reaction mix containing 10 μCi [γ-^{32}P]ATP (~3,000 Ci/mmol; Amersham), 25 mM unlabeled ATP, and 200 ng Rb protein as substrate were added to each sample and incubated at 30°C for 15 min. GST-Rb was expressed and purified as previously described [Meyerson and Harlow, 1994]. Kinase reactions were stopped by the addition of an equal volume of 2× SDS sample buffer [4% SDS, 150 mM Tris-HCl (pH 6.8), 20% glycerol, 0.02% bromophenol blue, 2 mM sodium vanadate] and by boiling for 5 min. Proteins were separated by electrophoresis in 10% SDS-PAGE; gels were dried and then autoradiograped.

Antibodies

The monoclonal and polyclonal antibodies anti-cyclin D1 (HD11), anti-PCNA (PC10), anti-p21 (F-5), anti-cdk4 (H-303), and anti-cdk6 (H-230 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal *MCT-1* antibodies were generated by inoculating rabbits with a synthetic peptide corresponding to the first 20 amino acids at the N-terminus of MCT-1. The immune sera was provided by Research Genetics (Huntsville, AL).

Western Blot

Cells in culture (5–10 × 10^6) were washed three times with PBS. Cell pellet was lysed with lysate buffer; 10 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 150 mM PMSF (Sigma). Total protein concentration in each sample was determined using a micro BCA method (Pierce), according to the manufacturer's instructions; 25 μg of total protein per sample was fractionated by electrophoresis in a tris-glycine PAGE gel (Novex, San Diego, CA). Under denaturing, reducing conditions. Transfer to supported nitrocellulose paper was performed using a Millipore electroblotting apparatus (Millipore, Marlborough, MA). Replicate filters were incubated either with anti-cyclin D1 antibody or *MCT-1* immune sera. Chemiluminescence was performed with ECL (Amersham Life Science, Arlington Heights, IL) according to the manufacturer's instruction. The filters were then exposed to X-ray film and bands were quantitated by laser densitometry using a personal densitometer (Molecular Dynamics, Sunnyvale, CA).

Focus Forming Assay

Stable transfectants and parent cell line were grown to near-confluence then plated in 100-mm tissue culture dishes at a density of 0.5 × 10^6 cells. The cultures were refed every 5–6 days, the number of transformed foci was determined 2 weeks later. Focus formation and morphologic changes were visualized both by the naked eye and by microscopy after a 1-h incubation with Coomassie blue. All experiments were reproduced at least three times with each DNA transfected.

RESULTS AND DISCUSSION

The progression of cells through the late G1 phase restriction point is controlled by G1 cyclins, including D- and E-type cyclins and their cognate cyclin-dependent kinases [Sherr et al., 1994]. The D type cyclins form complexes with either cdk4 or cdk6 [Baldin et al., 1993]. Overexpression of cyclin D1 can shorten the G1 interval of the cell cycle, reduce cell size and transform cells both in vitro and in vivo [Jiang et al., 1993; Lovec et al., 1993]. In a similar manner, cells constitutively expressing *MCT-1* have a shortened G1 interval and can grow in an anchorage-independent manner. We therefore wished to examine the potential impact of *MCT-1* overexpression on protein kinase-mediated G1 phase checkpoints. Employing NIH 3T3 fibroblasts constitutively expressing *MCT-1*, we examined the kinase activity of two cyclin D1-associated cdks: cdk4 and cdk6. Furthermore we determined the level of cyclin D1 protein as well as its subunit association with cdk4, cdk6, PCNA, and p21.

The steady-state protein levels of *MCT-1* in NIH3T3 cell lines stably transfected with pCMV-*MCT-1* or control vector (pCMV) as well as the parent cell line were analyzed using Western blot analysis. We observed a 20-kd band of increased intensity in cell lysates from NIH3T3 fibroblasts transfected with pCMV-*MCT-1* by Western blotting analysis (Fig. 1). Similar results were obtained in a transient transfection assay using a hemaglutinin (HA) tagged protein. We were able to immunoprecipitate HA-tagged *MCT-1* in transfected NIH3T3 fibroblasts, identifying a band at 20 kd only in cells expressing HA-tagged MCT-1 (Fig. 1). There was an increased level of cyclin D1 protein immunoprecipitated in asynchronously growing NIH3T3 cell lines transfected with

pCMV-*MCT-1* relative to controls (Fig. 2). Since both cdk4 and cdk6 associate with cyclin D1 during cell cycle progression we investigated the complex formation of these molecules, using co-immunoprecipitation analysis. As demonstrated in Figure 2, there was increased subunit complex formation in those cell lines constitutively expressing *MCT-1* relative to controls. Furthermore, we analyzed the physical interaction of PCNA with these complexes. Co-immunoprecipitation of cyclin D1 with PCNA revealed an increase in this interaction as well (Fig. 2). There was no direct physical interaction detected between *MCT-1* and these molecules under our assay conditions (data not shown).

Previous studies have demonstrated that ectopic expression of cyclin D1 can induce transcriptional activation of the p21 gene [Hiyama et al., 1997]. In normal human fibroblasts, the cdk inhibitory protein p21 is found in association with various cyclin/cdk complexes in combination with PCNA [Zhang et al., 1993]. Therefore, we examined these G1 cyclin/cdk complexes for p21. We found p21 to be associated with these complexes in those cell lines constitutively expressing *MCT-1* (Fig. 3). Previous studies have established that p21 can act as a universal inhibitor of cyclin/cdk kinase activity [Xiong et al., 1993]. Since increased G1 cyclin/cdk complexes were observed in our *MCT-1* overexpressing cell lines we were interested in assaying the catalytic activity of cdk4 and cdk6 in these cell extracts. As shown in Figure 4, a markedly increased ability to phosphorylate Rb substrate is observed when in vitro immune complex kinase assays were carried out with either cdk4 or cdk6 immunoprecipitated from the MCT-1 overexpressing cell lines. This supports a model in which cyclin/cdk complexes containing a single p21 molecule are not inhibited, but instead allows the stable complex formation with cyclin D1, cdk and PCNA [Zhang et al., 1994; Harper et al., 1995].

Using a focus forming assay, we demonstrated the ability of *MCT-1* overexpressing cell lines (pCMV-*MCT-1*) to form foci composed of smaller cells that grew in clusters when compared to the parent NIH3T3 and vector alone (pCMV) transfected cells (Fig. 5). These results are consistent with earlier work showing that fibroblasts overexpressing cyclin D1 have morphological changes and also grow in clusters [Jiang et al., 1993; Wang et al., 1994].

Finally, we were interested in determining whether T-cell malignancies would show an association between MCT-1 and cyclin D1 protein levels. As demonstrated in Figure 6, a number of asynchronously growing T-cell tumor cell lines show elevated *MCT-1* protein levels relative to PBL controls. This increased *MCT-1* protein expression correlated with levels of cyclin D1. Interestingly, the two IL-2-dependent cell lines NII85 and N1186 while having increased levels of cyclin D1 had no detectable MCT-1 protein. The HUT 78 cell line

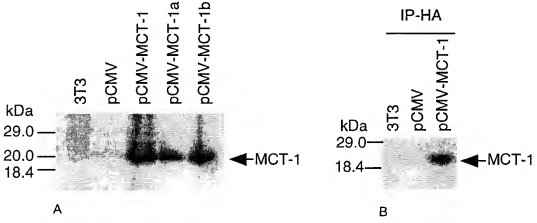

Fig. 1. **A:** Western Blot Analysis of stable cell lines. Steady-state *MCT-1* protein levels from equal amounts of whole cell lysate (50 μg) from the indicated cell lines were determined. pCMV-*MCT-1*, pCMV-*MCT-1*α, and pCMV-MCT-1β are NIH3T3 fibroblasts constitutively expressing MCT-1 (pCMV-*MCT-1*α-β are clonal derivatives obtained by limiting dilution). 3T3 and pCMV are the parent cell line and vector control transfectant, respectively. **B:** Analysis of transient transfectants. After immunoprecipitating with HA antibody we identified a 20 kd band by Western blot only in the IP lysate from pcDNA-HA-*MCT-1*-transfected NIH3T3 using an anti-*MCT-1* polyclonal sera.

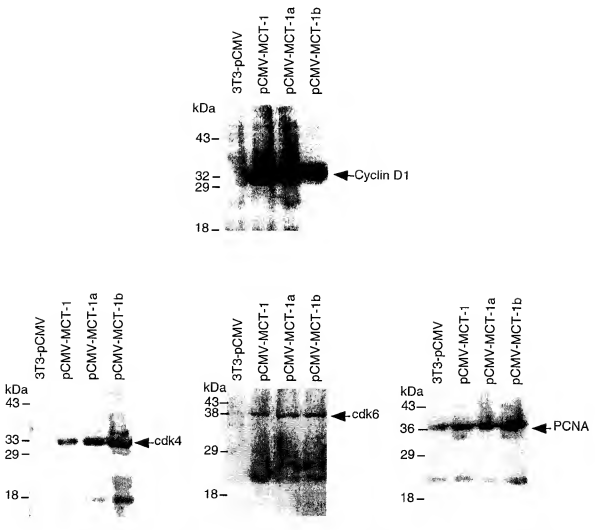

Fig. 2. Top, immunoprecipitation of cyclin D1 from MCT-1-transformed NIH3T3 fibroblasts. All *MCT-1* transfectant's demonstrated increased levels of cyclin D1 protein relative to controls. Lower, co-immunoprecipitation analysis of cyclin D1 with cdk4, cdk6 and PCNA. Briefly, whole cell lysates (100 μg) from *MCT-1*-expressing cell lines were immunoprecipitated with cyclin D1 antibody then the immunoprecipitate was immunoblotted with the indicated antibodies as above.

had the highest level of *MCT-1* protein consistent with gene amplification as previously described [Prosniak et al., 1998]. None of the other tumor cell lines analyzed in this study had *MCT-1* gene amplification (data not shown).

A striking finding of this study is the strong correlation between *MCT-1* overexpression and elevated cyclin D1 protein levels both in transfected NIH3T3 and in a panel of T-cell malignancies. For the first time, we demonstrate the endogenous expression of MCT-1 protein. Furthermore, we also show the biological significance of the genomic amplification of *MCT-1* in the HUT 78 cell line, since the level of *MCT-1* protein is greatly increased relative to the other cell lines without genomic amplification. Our data are consistent with *MCT-1* potentially acting through an upstream mechanism(s) involving cyclin D1 resulting in the dysregulation of G1 cdk activity. It is still unclear how *MCT-1* overexpression pushes cells through G1 but two facts are known. When cyclin D1 levels are elevated as in cells constitutively expressing *MCT-1*, several genes involved in growth regulation have been shown to be induced as well [Jiang et al., 1993]. The amino terminus of *MCT-1* has a region of homology with the C-terminal region of cyclin H [Prosniak et al., 1998], a region important for protein-protein interactions [Andersen et al., 1997]. Cyclin H forms a

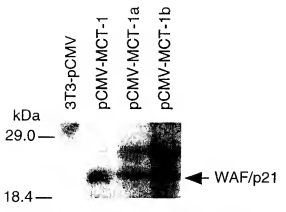

Fig. 3. Co-immunoprecipitation analysis of the cyclin/cdk complex in *MCT-1*-overexpressing cell lines for p21. After immunoprecipitating cyclin D1, immunoblotting with an antibody to p21 revealed complex formation between the G1 cyclin/cdk subunits and p21. All *MCT-1* overexpressing cell lines had increased p21 binding to the cyclin/cdk complex relative to controls.

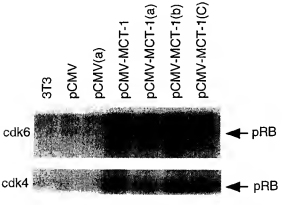

Fig. 4. Immune complex kinase assay: Extracts (50 μg) of indicated cell lines were immunoprecipitated with either cdk4 or cdk6 antibodies. Immune complexes were assayed for pRb kinase activity. Increased kinase activity in vitro was observed in all extracts of pCMV-*MCT-1*-transfected clones and mass culture.

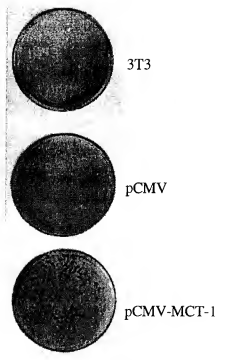

Fig. 5. Focus forming assay. The number of transformed foci was determined 2 weeks after plating at low density in 100-mm plates. Representative coomassie stained plates showing numerous induced foci in the *MCT-1* transfectant's plate, no foci were observed in the parent cell line or vector control. Morphologically transformed foci were evident at 10 days after plating.

ternary complex with cdk7 and MAT1, together they form the cdk-activating kinase (CAK). This CAK is responsible for activating cdk1, cdk2 and cdk4 [reviewed in Nigg, 1996]. A plausible explanation for the rapid G1 progression in *MCT-1* overexpressing cells is an enhancement of the CAK activity in addition to the increased cyclin D1 protein expression. This explanation is still hypothetical and additional experiments are ongoing in our laboratory to determine whether CAK activity is actually enhanced in cells overexpressing *MCT-1*. Furthermore, cyclin D/cdk4-cdk6 complexes are targeted by both the cip/kip family of CDIs as well as the INK family. The potential interaction of *MCT-1* with these molecules is an area of active investigation. Overexpression of *MCT-1* results in loss of normal cell cycle regulatory controls with an increase in G1 cyclin/cdk complex formation. While the underlying mechanisms are unclear at present, dysregulation of MCT-1 appears to be a potent transforming event in vitro when constitutively expressed and its level of expression is increased in T-cell malignancies relative to normal lymphocytes. These observations support further investigation in other human malignancies to verify its oncogenic potential. The fact that IL-2-dependent T-cell lines show elevated cyclin D1 in the absence of detectable *MCT-1* protein suggests that G1 mitogens such as IL-2 can push cells through G1/S without requiring the involvement of *MCT-1*. The physiological signals that regulate the level and function of *MCT-1* in normal as well as transformed cells remain to be established.

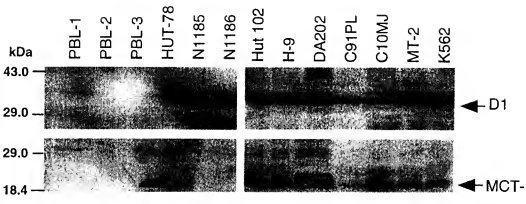

Fig. 6. Western blot analysis of T-cell lines. Cell lysates from asynchronously growing lymphoid cell lines were examined for steady-state *MCT-1* and cyclin D1 protein levels. The IL-2 independent cell lines showed an increase in *MCT-1* protein levels relative to the three normal donor PBL samples with the exception of C91PL. This was associated with an elevated cyclin D1 protein level. Observe the greatly increased *MCT-1* signal present in the HUT 78 lane, HUT 78 having an amplified *MCT-1* gene. The K562 leukemia cell line is a leukemia cell line derived from a CML in blast crisis.

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